

University of Groningen

Expression of porcine pancreatic phospholipase A2. Generation of active enzyme by sequence-specific cleavage of a hybrid protein from Escherichia coli

Geus, P. de; Bergh, C.J. van den; Kuipers, O.; Verheij, H.M.; Hoekstra, W.P.M.; Haas, G.H. de

Published in:
Nucleic Acids Research

DOI:
[10.1093/nar/15.9.3743](https://doi.org/10.1093/nar/15.9.3743)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1987

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Geus, P. D., Bergh, C. J. V. D., Kuipers, O., Verheij, H. M., Hoekstra, W. P. M., & Haas, G. H. D. (1987). Expression of porcine pancreatic phospholipase A2. Generation of active enzyme by sequence-specific cleavage of a hybrid protein from Escherichia coli. *Nucleic Acids Research*, 15(9).
<https://doi.org/10.1093/nar/15.9.3743>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Expression of porcine pancreatic phospholipase A₂. Generation of active enzyme by sequence-specific cleavage of a hybrid protein from *Escherichia coli*

P.de Geus*, C.J.van den Bergh, O.Kuipers, H.M.Verheij, W.P.M.Hoekstra⁺ and G.H.de Haas

Departments of Biochemistry and ⁺Molecular Cell Biology, State University of Utrecht, Padualaan 8, University Centre De Uithof, 3584 CH Utrecht, The Netherlands

Received April 22, 1986; Revised and Accepted April 10, 1987

Accession no. Y00146

ABSTRACT

The cDNA coding for the porcine pancreatic prophospholipase A₂ (proPLA) has been cloned and expressed in *E. coli*. Expression of proPLA could only be obtained in the form of intracellular aggregates after fusing the 15 kDa proPLA to a large (≥45 kDa) bacterial peptide. The fusion protein was readily purified from cell lysates, and specifically cleaved. Cleavage of the fusion protein was achieved with either hydroxylamine (at Asn/Gly sequences in the denatured protein), or trypsin (between the pro- and the mature PLA in the renatured fusion protein). The former method releases a proPLA-like enzyme, while the latter directly yields PLA. Renaturation of the fusion protein was made possible by the use of a recently reported new S-sulphonation method. The released (pro)PLA was purified (yields of 2-3 mg/ltr of culture medium), and showed identical properties compared to native (pro)PLA.

INTRODUCTION

One of the major goals in today's biochemistry is the design and construction of specifically tailored enzymes. Protein engineering has proven an important tool in this respect, both for the elucidation of the mechanisms that underly enzymatic catalysis, as well as for the defined construction of altered enzymes (see ref.1 for a review). However, the number of (eukaryotic) enzymes subjected to protein engineering appears to be somewhat small [1], as a result, probably, of two limitations: (i) the enzyme's kinetics and three-dimensional (3-D) structure should preferably be known in detail [1]; and (ii) the enzyme should be amenable to genetic manipulation, which includes expression of enzymatically active protein.

This latter factor seems to be the major cause for the observed low numbers of enzyme engineering projects. A recent review on the expression of eukaryotic proteins in *E. coli* [2]

lists but 5 enzymes, out of a total of 33 eukaryotic proteins expressed in the bacterial cytoplasm. Numerous other expression systems, both prokaryotic and eukaryotic, have been developed for the expression of biologically active proteins. Most of these are designed to secrete the protein, in order to obtain correct N-terminal residues, glycosylation and/or disulphide bonds [2-5], which appear to be the major difficulties associated with intracellular expression in E.coli [2]. Some of these systems have been used for protein engineering [6], but none of them seems to equal E.coli yet in its potential to produce gram amounts of intracellular recombinant polypeptide per liter of culture medium [2,7], in an easy and very rapid way.

In order to carry out a protein engineering project of lipolytic enzymes, we have devised and optimized an intracellular expression system in E.coli, that conveniently combines several published methods for: (i) purification of insoluble fusion proteins from the cytoplasm [2], (ii) S-sulphonation and subsequent reoxidation/renaturation of recombinant proteins [8-10], and (iii) site specific cleavage of fusion proteins with hydroxylamine [5,11], or enzymes, for the liberation of the peptide of interest. We have used this system for the expression of a pancreatic phospholipase A₂ (EC.3.1.1.4., abbreviated PLA), because it is the only lipolytic enzyme, that has been characterized to such detail [see ref. 12-14 for reviews] to allow for significant protein engineering to be done. It is a small (14 kDa) stable protein, containing seven disulphide bridges, which is secreted by the pancreas as a seven amino acid N-terminally extended proenzyme. In the intestine this proenzyme is converted by trypsin to the active enzyme [12]. The 3-D structure is known to 1.7 and 2.3 Å resolution for the bovine and porcine enzymes respectively [13], and extensive kinetic and sequence data are available for a large number of very homologous enzymes from various species [12].

In this report, we describe the cloning of cDNA, coding for porcine proPLA, and subsequently the various possibilities of our expression system, that lead to the rapid production of,

either mature PLA, or proPLA [12]. Quantitative data are presented for these methods. Milligram amounts of active PLA per liter of culture medium, representing relative yields of about 30%, were readily obtained. We discuss the potential use of the methodology for other heterologous proteins.

MATERIALS AND METHODS

Materials

All enzymes, oligo (dT), and oligo (dT)-cellulose were purchased from Pharmacia and used according to the manufacturer's instructions. β -Cyano-ethylphosphoramidite nucleotides for oligonucleotide synthesis were from Biosyntech (Hamburg, W-Germany). $\alpha^{35}\text{S}$ -dATP was obtained from Amersham. Guanidine hydrochloride and hydroxylamine were from Merck. Immunochemicals and TPCK-trypsin were obtained from Sigma. Synthetic phospholipids were kindly provided by R. Dijkman from our laboratory.

Strains and plasmids

E.coli K-12 Strains HB101 [15], JM103 [16], and AB1157 [17], have been described elsewhere. pBR322 tailed with poly(dG) in the PstI site, was bought from BRL. The pEX vector series was kindly made available to us by Dr. K. Stanley [18]. Plasmid pCI857, carrying the temperature sensitive λ -repressor with a pACYC origin was a gift of Dr. E. Remaut.

cDNA preparation and isolation of PLA clone

A fresh porcine pancreas was frozen and ground in a mortar filled with liquid nitrogen, and subsequently lyophilized. From the resulting fine powder, RNA was purified basically following the procedure of Chirgwin et al. [19]. mRNA was enriched on a poly(dT)-cellulose column according to Aviv and Leder [20], and was checked for biological activity by translating 250 ng in a wheat germ assay [21]. ^{35}S -Labeled proteins were visualized by SDS polyacrylamide gel electrophoresis [22] (SDS-PAGE) and fluorography. mRNA of sufficient quality was then used to prepare cDNA as described by Gubler and Hoffman [23]. The final double stranded cDNA material was cloned into pBR322 by the dC/dG tailing method. This material was used to transform HB101 [15] to tetracycline resistance. Ampicillin sensitive

colonies were replica plated onto nitrocellulose filters and screened for the presence of PLA coding sequences with the ^{32}P -labeled oligonucleotide mixture:

5'-ATGGA(T/C)TT(T/C)AA(T/C)AA(T/C)TA(T/C)GG-3', following standard procedures [24]. Hybrid selected translation was used to confirm the identity of the positive colonies [25]. The final cDNA was sequenced with a set of progressive sequence primers, synthesized by the phosphoramidite method [26]. The sequencing method of Sanger et al. [27] was used either on M-13 single stranded DNA, or on linearized double stranded DNA [28].

DNA techniques

Standard DNA operations were performed as described [29]. Unphosphorylated adaptor DNA molecules were ligated in a 100-fold molar excess to the vector ends, followed by precipitation with 0.1 volume of 5M sodium perchlorate and 0.5 volume of isopropanol for 15 min at room temperature. The latter technique ensures the complete removal of the excess unligated adaptor, which might adversely affect transformation efficiencies. The "adapted" ends were then phosphorylated and used for further cloning experiments. We routinely isolated restriction fragments from gel slices, using isotachopheresis [30].

Immunological techniques

PLA specific IgG was isolated from the serum of immunized rabbits by passing the serum through a Sepharose CL-4B column to which PLA had been covalently attached using CNBr [31]. Western blotting was performed essentially as described by Burnette [32]. Phospholipase-IgG complexes were visualised with a second antibody, coupled to horse radish peroxidase (HRP). Colony immuno-screening was done as follows: (i) colonies were replica-plated onto nitrocellulose filters, either before, or after induction of the expression plasmids; (ii) lysis of the colonies was evoked by heating the filters on Whatman 3MM paper, soaked in 2% SDS, in a microwave oven for 30 sec at 600 W, followed by another 60 sec at 200 W; (iii) after precipitation of the antigens with 10% TCA for 15 min at 4°C, the filters were neutralized with 1M Tris and washed in saline; (iv) finally, the antigens were detected in the same way as in the western blotting procedure.

Phospholipase A2 assays

The proenzyme is active on monomeric substrate only, and is routinely assayed using dihexanoyl-dithio-phosphatidylcholine (thio-diC₆PC) as a substrate [33]. Monomeric assay conditions were: 0.5 mM thio-diC₆PC, 50 mM Tris (pH 8), and 20 mM CaCl₂ with 0.1 mM dithionitrobenzoic acid (Ellman's reagent). The enzymatic activity was continuously followed by recording the absorbance at 412 nm. Specific activity of the porcine PLA under these conditions is around 10 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$, enabling the determination of less than 0.1 μg of proPLA.

The active enzyme (after activation of the proenzyme by trypsin), is conveniently assayed in the pH-stat [12], using micellar L-dioctanoylphosphatidylcholine (L-diC₈PC) as the substrate. Micellar assay conditions were: 1 mM L-diC₈PC, 1 mM sodium borate (pH 8) and 15 mM CaCl₂ in a total volume of 2.5 ml. The fatty acids were titrated with 10 mM NaOH. In this system, porcine PLA exhibits a specific activity of about 2000 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$, consequently, 0.2 μg of PLA can be easily measured.

The renatured complete fusion protein was directly assayed for its content of active PLA, by adding a portion to the micellar assay reaction vessel, in which 10 μg of TPCK-trypsin was included. Within 5 to 10 minutes the recorded activity was linear with time, and this extrapolated value was compared to the value obtained in the same manner with proPLA.

Recovery of (pro)PLA from isolated fusion proteins

Proteins were harvested from AB1157 harbouring pCI857, and the appropriate expression plasmid, 3 hrs after the induction of expression (by temperature shift). Protein aggregates were isolated from cell lysates by a 30 min centrifugation at 5000xg [34], and washed once with 0.1 % of Triton-X100.

PLA was recovered as follows. Fusion protein was sulphonated at 2-5 mg/ml, as recently described by Tannhauser and Sheraga [10]. The S-sulfo protein was precipitated by dialysis against 1% HAC, and the precipitate was washed several times with water. Renaturation was induced by dissolving the protein, at 0.4 mg/ml, in 2 M Urea, 25 mM sodium borate (pH 8.5), 5 mM EDTA and a mixture of 2 mM reduced- and 1 mM oxidized glutathione.

Renaturation usually reached a maximal level after 24 hrs at room temperature, as measured in the L-diC₈PC assay. After dialysis and concentration, preparative tryptic digestion of the renatured fusion protein was done in a buffer containing: 1 mg/ml fusion protein, 20 mM Tris (pH 8), 15 mM CaCl₂, and 1 mM D-diC₈PC. Trypsin (15 µg/ml) was added at intervals during a 2-3 hr incubation at room temperature, until the PLA activity had reached a maximum. The mixture was then acidified to pH 5 and centrifuged to remove the insoluble peptides. PLA was finally purified on CM ion exchange resin, following established methods [12].

ProPLA was isolated from the fusion protein by cleavage with hydroxylamine [11]. First, the inclusion bodies were solubilized and reduced, at 5 mg/ml concentration, in 6 M Guanidine, 50 mM Tris (pH 8), 1 mM EDTA and 500 mM β-mercaptoethanol, for 1 hr under N₂. The mixture was then centrifuged and the supernatant acidified with 1% HAc and dialyzed against 0.1 % HAc. The precipitated fusion protein was washed twice with dilute acid, and dissolved in 6 M Guanidine, 2 M hydroxylamine, adjusted to pH 8.5 with LiOH, at a protein concentration of 5 mg/ml. After a 3½ hr incubation at 45 °C, the reaction was stopped by the addition of ten volumes of 30 % HAc (V/V), and the resulting peptide mixture was dialyzed against 1% HAc. Insoluble material was removed by centrifugation and the soluble fraction was adjusted to 1.5 M Guanidine, 50 mM Tris/HCl, 10 mM Ca²⁺ and 5 mM Cys, pH 8.3 [35]. Following renaturation and dialysis, proPLA activity could be directly measured.

RESULTS

Cloning and sequence of prophospholipase A2 encoding cDNA

mRNA from a fresh porcine pancreas was used for the preparation of a cDNA bank in *E. coli* HB101. PLA specific clones were identified, and one of them (PPG4) was sequenced, as described in Materials and Methods. It contained a 560 base pairs (bp) long cDNA insert, covering the complete coding region for the preproPLA. This coding region is shown in Fig 1. The predicted amino acid sequence is in full agreement with the one known for porcine proPLA [36]. As expected for a secreted

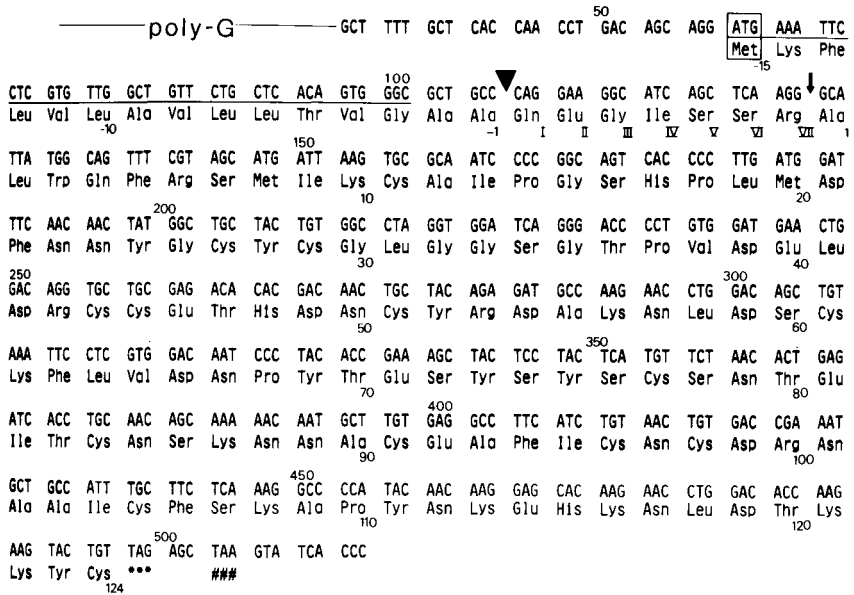


Fig 1. The DNA coding- and protein sequence of pig pancreatic preprophospholipase A2. The solid triangle represents the substrate site for signal peptidase. Roman numerals denote residues of the activation peptide in proPLA, which is naturally cleaved off by trypsin (arrow). The restriction map shows the signal and the activation peptide as the hatched and open areas respectively.

protein, we found a 15 amino acids N-terminal extension, matching the criteria for a signal peptide as outlined by Perlman and Halvorson [37]. This tentative signal peptide is also very homologous to the ones recently found in cloned PLA cDNA's from rat and dog pancreas and human lung [38,39]. Furthermore, expression experiments in eukaryotic cell lines, confirmed the functional integrity of this cDNA sequence, since the proPLA was accurately processed into the culture medium (P.de Geus, unpublished results).

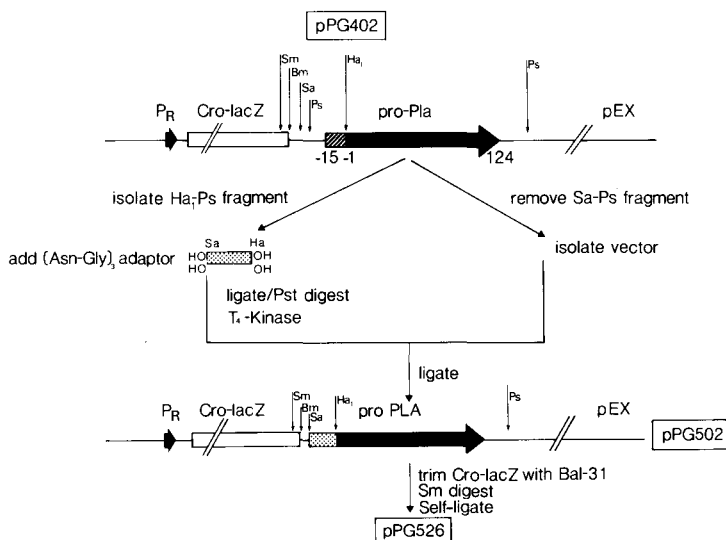


Fig 2. Construction of *cro/lacZ-proPLA* expression vectors with (Asn/Gly)₃ linkage cassette (dotted box). The hatched area represents the signal peptide. Residue numbering of *proPLA* refers to Fig 1. Abbreviations: *Sm*, *Sma*I; *Bm*, *Bam*HI; *Sa*, *Sa*I; *Ps*, *Pst*I; *Ha*, *Hae*II. The properties of the different vectors are described in the text.

Expression of *PLA* cDNA in *E. coli* K-12

As it became evident that *PLA* could not be expressed to any significant levels, either directly in the cytoplasm, or after processing into the periplasmic space (data not shown), we developed a strategy for the expression of *PLA* as a fusion protein. This approach has led in several cases [2], to the successful expression of eukaryotic proteins in *E. coli*, but inevitably requires a cleavage step in the end. For this, we intended to use the natural tryptic cleavage site, at the junction of the propeptide and the native enzyme (cf. Fig.1).

The 560 bp cDNA fragment of pPG4 was cloned into the *pEX* vector series, and clones were screened for the production of *PLA* antigens by the colony immuno-screening method. One of these, pPG402 (Fig 2), produced large quantities of the expected 130 kDa *cro/lacZ-PLA* fusion protein (Fig 3, lanes a and b). Unfortunately, this protein, and also smaller deleted variants

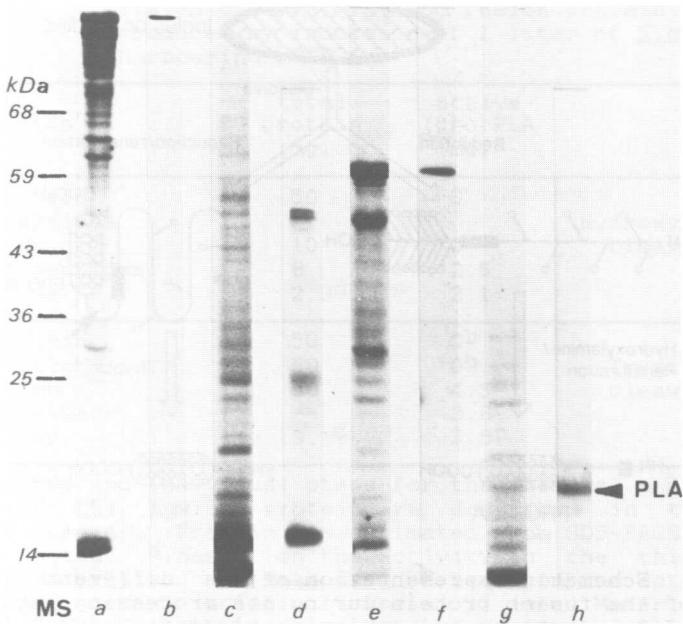


Fig 3. SDS-PAGE of isolated inclusion bodies containing PLA fusion protein, before and after hydroxylamine treatment. MS: Molecular weight standard. Lane a: fusion protein from pPG402. Lane c: hydroxylamine digest of a. Lane e: fusion protein from pPG526. Lane g: hydroxylamine digest of e. Lanes b,d,f,h: Western blots of respectively lanes a,c,e, and g, using anti-PLA antibodies. Proteins were stained with fast green. The different expression vectors are explained in the text.

(data not shown), could not be directly renatured nor cleaved by trypsin, due to solubility problems. To overcome this problem, we elaborated two independent alternative schemes: (i) chemical cleavage under denaturing conditions, and (ii) chemical modification to increase the solubility under renaturation conditions, allowing subsequent cleavage by trypsin. The order of individual steps, and protein folding stages, involved in both routes is schematically outlined in Figure 4. It can be seen in Fig.4 that by working out both routes the pro- as well as the mature enzyme can be obtained and studied.

Recovery of proPLA from the fusion protein by chemical cleavage

Hydroxylamine cleavage [11] of the peptide bond between Asn and Gly residues (absent in PLA), was tested, a priori, on the

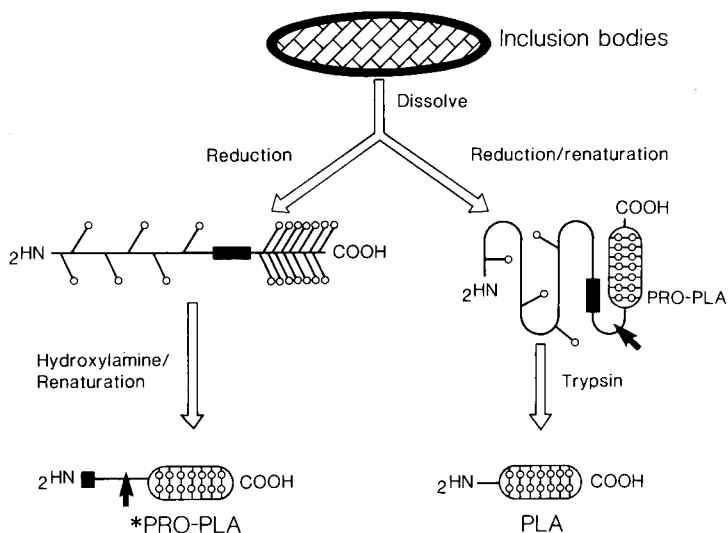


Fig 4. Schematic representation of the different (folding) stadia of the fusion protein during its processing into either proPLA (left part) by hydroxylamine cleavage, or PLA (right part) by S-sulphonation/renaturation and trypsin cleavage. —○: free -SH groups, —○—: S-S bridges, ■: {Asn/Gly}₃ linker. The arrow represents the cleavage site for trypsin. GlyAlaAla-extended proPLA (the product of the NH₂OH cleavage, arising from the location of the Asn/Gly linker in the HaeII site of the PLA cDNA), is denoted by *PRO-PLA.

fusion protein from pPG402, for its general sequence specificity. A digest of pPG402 protein (Fig 3 lane c) showed distinct peptide fragments, in agreement with the locations of the Asn-Gly sequences in the fusion protein. Besides the expected 25 kDa, PLA containing peptide, partial (larger) cleavage products can be seen in a Western blot (lane d), indicating a less efficient cleavage reaction at some of the Asn/Gly sites. The heavy band at the gel front is due to the accumulation of small peptide fragments, resulting from the aspecific cleavage by NH₂OH. Therefore, the complete procedure was optimized, with respect to proPLA yields, by: (i) insertion of a {Asn/Gly}₃ linker sequence in the HaeII site, between proPLA and the bacterial leader fragment of the fusion protein (Fig 2, pPG502), and (ii) by deleting as much as possible of the leader sequence, without affecting the final yield of fusion

Table I. Yields of (pro)PLA from fusion protein produced by induction of 1 liter of *E.coli* harbouring pPG526.

stage of purification	total protein (mg)	active (pro)PLA (mg) ^a	
fusion protein ...	50	0	Hydroxylamine cleavage
soluble peptides after NH ₂ OH ...	10	0	
renatured peptides.	8	2.5	
CM-cellulose ...	2.3 ^d	2.1	
fusion protein ...	50	0 ^b	Tryptic cleavage
S-sulphonation ...	50	0 ^b	
renaturation ...	50	4.5 ^b	
trypsin cleavage...	--	3.8 ^c	
CM-cellulose ...	3.1 ^d	2.8 ^c	

The procedures and individual steps for the chemical and tryptic cleavage of the fusion protein are described in the text (cf. also Figure 4). Protein was estimated from SDS-PAGE, unless otherwise noted. ^a:based on the activity in the thio-diC₈PC assay (see Materials and Methods). ^b:based on the activity in the L-diC₈PC assay including trypsin (see Materials and Methods). ^c:based on the direct activity in the L-diC₈PC assay, after preparative scale tryptic digestion of the fusion protein. ^d:based on the absorption at 280 nm.

protein too much. The resulting plasmid, pPG526 (Fig 2), upon induction, produced a 59 kDa fusion protein up to about 2% of total cellular protein, which could now be effectively digested with hydroxylamine (Fig 3, lanes e-h). The peptide material thus obtained from 1 ltr of cell culture, was renatured and the activity of proPLA directly measured. The results are summarized in Table I. It can be seen that the yield of active proPLA is about 20 % of the theoretically possible yield (on the basis of the molecular weight ratio: PLA/fused-PLA). The active proPLA was purified on CM-cellulose in the same manner as is described below for PLA.

Recovery of PLA from the fusion protein by tryptic cleavage

We found that the very rapid and mild S-sulphonation procedure, recently described [10], rendered the fusion protein from pPG526 soluble in renaturation buffer (see Materials and Methods). After S-sulphonation and dialysis, the fusion protein from 1 ltr of cell culture was dissolved in 8M Urea buffer, and renaturation was started by dilution to 2M Urea with

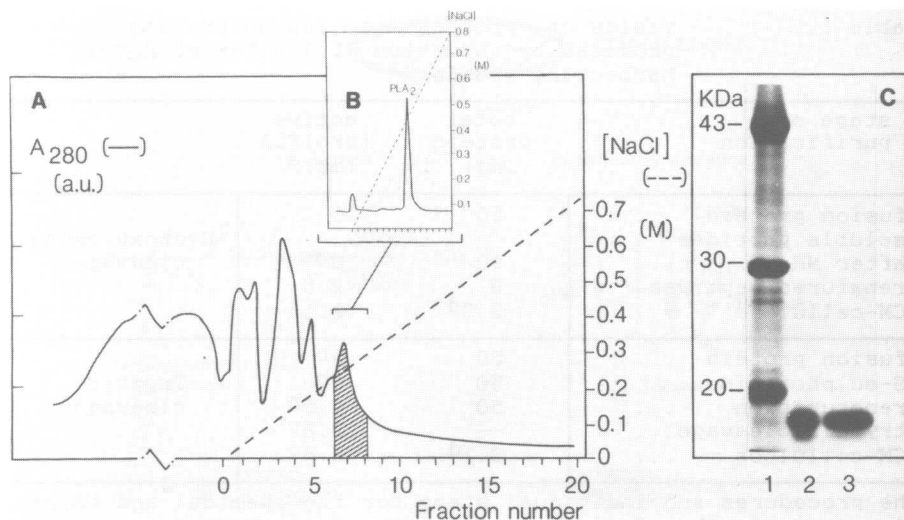


Fig 5. Purification of PLA after tryptic liberation of the enzyme from fusion protein. Panel A shows the elution pattern of a 10x2 cm CM-cellulose column, at 4°C and pH 5.3 in 10 mM acetate buffer, with a 2 x 100 ml salt gradient as indicated. a.u.: arbitrary units. Panel B: The PLA fraction (hatched area) was analyzed on the FPLC mono-S column (Pharmacia), using a 10 mM acetate buffer of pH 4.8. Panel C: SDS-PAGE of: lane 1, molecular weight standard; lane 2, purified pancreatic PLA; lane 3, purified PLA from CM column in panel A.

concomittant addition of the glutathione redox couple. No formation of a precipitate was observed during an overnight stand at room temperature, nor thereafter, during the dialysis against ammonium bicarbonate of pH 7.8. Thus, after concentration, the renatured protein could be cut with trypsin on a preparative scale, to yield the active PLA (30 % of theoretical yield, see Table I), which could be directly measured in the L-diC₈PC assay. After acidification of the reaction mixture a large precipitate formed, which did not include the PLA. The supernatant obtained after this treatment was loaded on a CM cellulose column at 4 °C and the proteins were eluted with a salt gradient as indicated in Figure 5. The PLA containing peak was collected and analyzed by FPLC mono-S, and SDS-PAGE (Fig.5). It behaved in the same way as native PLA purified from porcine pancreatic pancreas. The protein was

judged to be over 90 % pure, and this was confirmed by comparing the specific activity in the micellar assay (Table I). These data show that we have produced authentic PLA from E. coli derived fusion protein. Purification to near homogeneity is now in progress with a scaled up procedure.

DISCUSSION

The porcine PLA cDNA clone isolated here, represents the more predominant species of PLA isoenzymes found in pancreatic juice, namely the one with Ala₁₂, His₁₇, Met₂₀, and Glu₇₁, rather than Thr₁₂, Asp₁₇, Leu₂₀, and Asn₇₁ [40]. This is not surprising since the oligonucleotide used in the initial screening, coded for amino-acid Met₂₀-Gly₂₆. From the cDNA sequence it also becomes clear that the pyroglutamic acid residue found at the N-terminus of the natural proenzyme [12] must derive from glutamine initially synthesized at that position. This conversion can well have taken place in the secretory granules of the pancreatic cells.

Several different approaches have been taken to express proPLA in E. coli. Only when fused to a bacterial peptide could PLA be expressed to sufficient amounts, as an insoluble intracellular aggregate, which is not uncommon for eukaryotic proteins in E. coli [2,7]. There are currently scarce quantitative data on the necessary cleavage and renaturation steps involved with fusion proteins [2], nor is there a general methodology for solving these problems yet. In this report, we describe two different methods, one chemical and one enzymatic, for the site specific cleavage of the fusion protein. They were chosen so as to enable the isolation of both a proenzyme-like and the active enzyme molecule (cf. Fig.4). Although the chemical cleavage reaction yields GlyAlaAla- extended proPLA, the enzymatic function is identical to that of native proPLA, since the N-terminus does not play a functional role in the proenzyme [12]. The markedly different properties [12] of both enzyme species will be very helpful in the future for the interpretation of the properties of engineered PLAs.

The chemical cleavage by hydroxylamine is very convenient in eliminating bacterial proteases in an early stage of the

purification [cf ref.34]. The sequence specificity and yield were high, provided that the {AsnGly}₃ linker was put in front of proPLA. Therefore we think the method of general applicability to those cases where no intrinsic AsnGly sequences are present, and where either the N-terminus is not important for biological activity, or can be subsequently removed by another cleavage step.

Enzymatic cleavage methods of fusion proteins are clearly highly desirable because of their mildness and potentially high sequence specificity. The latter, however, tends to be displayed only, in full, in non-denaturing buffers and on structurally intact (folded) protein substrates. Fusion proteins, in most cases, will be hard to solubilize, thus preventing refolding of the passenger eukaryotic protein. We applied a new method of S-sulphonation of cysteine residues [10], which eventually overcomes this solubility problem by simultaneous removal of the sulfo-groups (which solubilized the unfolded protein) and promotion of S-S bridge formation (rendering the protein soluble). It enabled us to produce active PLA within two days following cell lysis, with a minimum of experimental work. The method, therefore, is potentially very attractive for other (cystein containing) proteins too, produced in E. coli.

The choice of proteolytic enzymes has been confined to a few very specific ones [2]. For instance, Nagai and Thogersen [41] were the first to use factor X_a for precise fusion protein cleavage. It recognizes a tetrapeptide sequence and therefore is claimed to be highly specific, even under denaturing conditions [42]. But the enzyme is expensive and needs incubation times of 3 to 24 days [42]. Furthermore, as for all proteases, the specificity is likely to depend also on the environment of the target peptide in the fusion. Probably for these reasons, Varadarajan et.al. used trypsin instead of factor X_a to release myoglobin from a fusion protein, although the linkage was through a X_a recognition sequence [7]. Also in the case of the fused proPLA, tryptic cleavage was observed to be about 5-fold slower than the comparable activation of native proPLA, indicating effects of the environment on the accessibility of the ArgvIIAla₁ (Fig.1) bond.

All of these data seem to argue that efforts in this field should be directed towards the introduction, into fusion proteins, of more highly labile peptidyl substrate bonds for existing specific proteolytic enzymes. Positioned in fusion proteins, these would then allow differential cleavage of fusion proteins leaving the protein of interest intact, despite the possible presence therein of other cleavage sites. In line with this, we are presently constructing, in pPG526, a Pro^{VI}Arg^{VI}Ala^I derivative of the PLA activation-peptide sequence, which is known, in horse pancreatic PLA, to be cleaved ten times faster than the corresponding site in the porcine enzyme (this laboratory, unpublished results).

We now have available a versatile set of expression tools with which the engineering of (pro)PLA can be undertaken. The methods described here could also be useful for the expression as fusion protein in *E. coli* of other proteins rich in disulphide bridges.

*To whom correspondence should be addressed

REFERENCES

1. Leatherbarrow, R.J. and Fersht, A.R. (1986) Protein Engineering 1, 7-16.
2. Marston, F.A.O., Biochem.J. (1986) 240, 1-12.
3. Nicaud, J.M., Mackman, N. and Holland, I.B. (1986) J. Biotechnol. 3, 255-270.
4. Bebbington, C. and Hentschel, L. (1985) Trends Biotech. 3, 314-317.
5. Abrahmsen, L., Moks, T., Nilsson, B. and Uhlen, M. (1986) Nucleic Acids Res., 14, 7487-7500.
6. Craik, C.S., Largman, C., Fletcher, T., Roczniaik, S., Barr, P.J., Fletterick, R., and Rutter, W.J. (1985) Science 228, 291-297.
7. Varadarajan, R., Szabo, A. and Boxes, S.G. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 5681-5684.
8. Goeddel, D.V., Kleid, D.G., Bolivar, F., Heyneker, H.L., Yansura, D.G., Crea, R., Hirose, T., Kraszewski, A., Itakura, K. and Riggs, A.D. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 106-110.
9. Cabilly, S., Riggs, A.D., Pande, H., Shirely, J.E., Holmes, W.E., Rey, M., Perry, L.J., Wetzell, R., and Heyneker, H.L. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 3273-3277.
10. Tannhäuser, T.W. and Scheraga, H.A. (1985) Biochemistry 24, 7681-7688.

11. Bornstein, P. and Balian, G. (1977) *Meth. Enzymol.* 47, 132-145.
12. Verheij, H.M., Slotboom, A.J. and de Haas, G.H. (1981) *Rev. Physiol. Biochem. Pharmacol.* 91, 91-203.
13. Dijkstra, B.W., Kalk, K.H., Hol, W.G.J. and Drenth, J. (1981) *J. Mol. Biol.* 147, 93-123.
14. Volwerk, J.J. and de Haas, G.H. (1982) in *Lipid-Protein Interactions*, Jost, P.C. and Griffith, O.H. Eds., Vol I, pp. 70-149, John Wiley & Sons.
15. Boyer, H.W. and Roulland-Dussoix, D. (1969) *J. Mol. Biol.* 41, 459-472.
16. Vieira, J. and Messing, J. (1982) *Gene* 19, 259-268.
17. De Witt, S.K. and Adelberg, E.A. (1962) *Genetics* 47, 577-585.
18. Stanley, K.K., and Luzio, J.P. (1984) *EMBO J.* 3, 1429-1434.
19. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, J. (1979) *Biochemistry* 18, 5294-5299.
20. Aviv, H. and Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* 69, 1408-1412.
21. Anderson, C.W., Straus, J.W. and Dudock, B.S. (1983) *Meth. Enzymol.* 101C, 635-644.
22. Lugtenberg, B., Meyers, J., Peters, R., van der Hoek, P. and van Alphen, L. (1975) *FEBS Lett.* 58, 254-258.
23. Gubler, U. and Hoffman, J. (1983) *Gene* 25, 263-269.
24. Wallace, R.B., Johnson, M.J., Hirose, T., Miyake, T., Kawashima, E.H. and Itakura, K. (1981) *Nucl. Acids Res.* 9, 879-894.
25. Parnes, J.R., Velan, B., Felsenfeld, A., Ramanathan, L., Ferrini, U., Appella, E. and Seidman, J.G. (1981) *Proc. Natl. Acad. Sci. USA* 78, 2253-2257.
26. Sinha, N.D., Biernat, J. and Köster, H. (1983) *Tetrahedron Lett.* 24, 5843-5846.
27. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* 74, 5463-5467.
28. Wallace, R.B., Johnson, M.J., Suggs, S.V., Miyoshi, K., Bhatt, R., and Itakura, K. (1981) *Gene* 16, 21-26.
29. Maniatis, R., Fritsch, E.F. and Sambrook, J. (1982), *Molecular Cloning. A laboratory Manual*, Cold Spring Harbor Laboratory Press, New York.
30. Ofverstedt, L., Hammarström, K., Balgobin, N., Hjertén, S., Pettersson, U. and Chattopadhyaya, J. (1984) *Biochim. Biophys. Acta* 782, 120-126.
31. Meyer, H., Meddens, M., Dijkman, R., Slotboom, A.J. and de Haas, G.H. (1978) *J. Biol. Chem.* 253, 8564-8569.
32. Burnette, W.N. (1981) *Anal. Biochem.* 112, 195-203.
33. Aarsman, A.J., van Deenen, L.L.M. and van den Bosch, H. (1976) *Bioorg. Chem.* 5, 241-247.
34. Winkler, M.E. and Blaber, M. (1986) *Biochemistry* 25, 4041-4045.
35. van Scharrenburg, G.J.M., de Haas, G.H. and Slotboom, A.J. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* 361, 571-576.
36. Puijk, W.C., Verheij, H.M., de Haas, G.H. (1977) *Biochim. Biophys. Acta* 492, 254-259.
37. Perlman, D. and Halvorson, H.O. (1983) *J. Mol. Biol.* 167, 391-409.

38. Ohara, O., Tamaki, M., Nakamura, E., Tsuruta, Y., Fujii, Y., Shin, M., Teraoka, H. and Okamoto, M. (1986) *J. Biochem.* 99, 733-739.
39. Seilhamer, J.J., Randall, T.L., Miles, Y. and Johnson, L.K. (1986) *DNA* 5, 519-527.
40. Puijk, W.C., Verheij, H.M., Wietzes, P. and de Haas, G.H. (1979) *Biochim. Biophys. Acta* 580, 411-415.
41. Nagai, K. and Thogersen, H.C. (1984) *Nature* 309, 810-812.
42. Nambiar, K.P., Stackhouse, J., Presnell, S.R. and Benner, S.A. (1987), *Eur. J. Biochem.* 163, 67-71.